JOURNAL OF THE AMERICAN WATER RESOURCES ASSOCIATION
DECEMBER AMERICAN WATER RESOURCES ASSOCIATION 2005

ESCHERICHIA COLI SURVIVAL IN MANTLED KARST SPRINGS AND STREAMS, NORTHWEST ARKANSAS OZARKS, USA[1]

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ABSTRACT: Recent studies indicate fecal coliform bacterial concentrations, including *Escherichia coli* (*E. coli*), characteristically vary by several orders of magnitude, depending on the hydrology of storm recharge and discharge. *E. coli* concentrations in spring water increase rapidly during the rising limb of a storm hydrograph, peak prior to or coincident with the peak of the storm pulse, and decline rapidly, well before the recession of the storm hydrograph. This suggests *E. coli* are associated with resuspension of sediment during the onset of turbulent flow, and indicates viable bacteria reside within the spring and stream sediments. *E. coli* inoculated chambers were placed in spring and stream environments within the mantled karst of northwest Arkansas to assess long term (> 75 days) *E. coli* viability. During the 75-day study, a 4-log die-off of *E. coli* was observed for chambers placed in the Illinois River, and a 5-log die-off for chambers placed in Copperhead Spring. Extrapolation of the regression line for each environment indicates *E. coli* concentration would reach 1 most probable number (MPN)/100 g sediment at Copperhead Spring in about 105 days, and about 135 days in the Illinois River, based on a starting inoculation of 2.5 x 107 MPN *E. coli*/100 g of sediment. These *in situ* observations indicate it is possible for *E. coli* to survive in these environments for at least four months with no fresh external inputs.

(KEY TERMS: water quality; nonpoint source pollution; Ozarks; mantled karst aquifers; fecal coliform bacterial survival; *Escherichia coli*.)

Davis, Ralph K., Sherri Hamilton, and John Van Brahana, 2005. *Escherichia coli* Survival in Mantled Karst Springs and Streams, Northwest Arkansas Ozarks, USA. Journal of the American Water Resources Association (JAWRA) 41(6):1279-1287.

INTRODUCTION

The mantled karst terrain of northwest Arkansas is an area of intense animal production, in part because of favorable climate, the availability of open pasture land, and the general poor suitability of the soil to support row crops. Animal manure (associated primarily with poultry, cattle, and swine production) is beneficially applied as fertilizer on pasture land in the region. However, because the mantled karst is characterized by highly fractured, dissolutionally modified limestone, surface water can greatly influence ground water quality. These water quality impacts include nutrient, bacterial, and hormonal loading, among others (Davis *et al.,* 2000; Peterson *et al.,* 2000, 2001, 2002).

Concentrations of fecal coliform bacteria, including *Escherichia coli* (*E. coli*), have been shown to vary several orders of magnitude [10 colony forming units (cfu)/100 ml to over 50,000 cfu/100 ml] in water discharging from springs in this area in response to storm inputs (Davis *et al.,* 2000; Peterson *et al.,* 2001). Additionally, water quality data collected from streams and springs in northwest Arkansas suggest that concentrations of fecal coliform bacteria and *E. coli* increase rapidly during the rising limb of a storm hydrograph, peak prior to or coincident with the peak of the storm pulse and decline rapidly, well before the recessional portion of the storm hydrograph (Davis *et al.,* 2000; Marshall *et al.,* 1998; Peterson *et al.,* 2001). This pattern suggests that the fecal bacteria transported through these karst aquifers and in the streams are resuspended along with sediment during the onset of turbulent flow. This leads to the conclusion that fecal coliform bacteria and *E. coli* are in residence within the sediments and can survive in these environments for perhaps several months. This

[1]Paper No. 03134 of the *Journal of the American Water Resources Association* (JAWRA) (Copyright © 2005). **Discussions are open until June 1, 2006.**

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is generally the longest period that would occur between episodes of fresh fecal bacterial input from surface sources, (Davis *et al.,* 2000; Marshall *et al.,* 1998; Peterson *et al.,* 2001). Davies and Bavor (2000) also conclude that wetland and pond sediments act as a reservoir of viable bacteria that may be resuspended back into the water column by storm activity. Potential sources of fecal bacterial inputs in this area include bats (in caves), human septic effluent, cattle, swine, poultry, and other wild, warm blooded animals (deer, turkey, bear, small game) (Davis *et al.,* 2000; Marshall *et al.,* 1998; Edwards *et al.,* 1997). Because there are multiple potential sources, and because large quantities of animal manures are applied as fertilizer, it is imperative that the fate and transport of these organisms in the surface water and ground water of these mantled karst environments are understood. Therefore, the main objective of this project was to assess the die-off rate of *E. coli* in the sediments of springs and streams in the mantled karst of northwest Arkansas. This objective addresses one component of the fate of these organisms in these types of environments.

E. coli normally live in the intestines of warm-blooded animals. *E. coli* make up a large percentage of fecal coliform bacteria, and its occurrence in water and sediment is generally an indication of contamination by fecal matter (Chapelle, 2001). They are often used as a pathogen indicator in water because they can be readily detected, providing a surrogate for assessing impact by other potentially pathogenic microorganisms associated with fecal contamination (Berg, 1978; Berg, 2000; Francy *et al.,* 1993; Elmund *et al.*, 1999). While not necessarily posing a direct health threat certain strains of *E. coli*, such as O157:H7, can cause serious health problems. Contamination of ground water by O157:H7 recently proved to be fatal in Walkerton, Ontario, Canada (Brooke, 2000; Ali, 2004). *E. coli* are a member of the *Enterobactericeae* (the intestinal bacteria) and belong to the order *Eubacteria* (Berg, 1978; Berg, 2000; Chapelle, 2001). These bacteria are facultatively anaerobic, gram negative rods that can grow under both aerobic and anaerobic conditions (Chapelle, 2001). If molecular oxygen is available, the bacteria rely on respiratory metabolism to survive. In the absence of molecular oxygen, the organisms use fermentation as an alternate means of survival (Berg, 2000; Chapelle, 2001). Once *E. coli* enter receiving waters, their population may show a net increase (reproduction exceeds die-off), remain static (reproduction equals die-off), or show a net decrease (reproduction is less than die-off), depending on the conditions of the environment.

Since the optimum temperature for reproduction is in the intestines of warm blooded animals, one would assume a relatively rapid die-off once outside this optimum environment. However, several studies show that fecal coliform bacteria survive for extended periods outside their optimum environment. Flint (1987) found that *E. coli* introduced into autoclaved, filtered river water can survive at temperatures from 4˚C to 25˚C for up to 260 days. The survival time recorded by Flint is longer than other studies and may be due in part to a lack of predation in the sterile environment and the fixed temperatures of his study. Van Donsel *et al.* (1967) reported that a 90 percent reduction of bacteria occurred within soil over three days in the summer and 15 days in winter. A more recent study by Teague *et al.* (1995) found a 99.9 percent reduction of *E. coli* in soil at 35˚C in 19 days, whereas the same reduction at 5˚C took 38 days. A study of *E. coli* in marine sediment shows that the bacteria remained viable for 68 days and suggested that the sediment "provides a favorable, nonstarvation environment for the bacteria" (Davies *et al.,* 1995). Schumaker (2003) reports that significant numbers of fecal bacteria can survive for as much as eight weeks after treatment of test plots with poultry litter. Based on several of these studies, it is clear that lower temperature environments reduce the metabolism of the bacteria, and effectively increase their life span, ultimately resulting in survival for extended periods in less than optimum conditions.

FIELD SITE

The site used to conduct this field experiment was the Savoy Experimental Watershed (SEW) (Figure 1). The SEW is approximately 1,250 hectares located about 24 km west of the University of Arkansas campus. It was selected as the study site because it is representative of mantled karst aquifers throughout northwest Arkansas and much of the Ozarks. The Mississippian Boone and St. Joe formations form the Springfield Plateau aquifer, a shallow, mantled karst aquifer characterized by a high degree of surface water and ground water interaction (Brahana *et al.,* 1999; Imes and Emmet, 1994). The Chattanooga Shale is a confining unit that separates this shallow aquifer from the deeper Ozark aquifer. The SEW has a number of springs that are typical of those found throughout the Springfield Plateau of the Ozarks. Copperhead Spring was selected as the representative Ozark spring environment. The Illinois River, which borders the site, was selected as a representative Ozark stream environment.

Figure 1. Location of Savoy Experimental Watershed (SEW) Within Arkansas (site shown as star).

METHODS

Survival chambers inoculated with *E. coli* were placed in a spring and stream environment in the mantled karst terrain of northwest Arkansas to assess the viability of this organism in these environments over extended periods (as many as 75 days). The chambers were constructed to provide an environment similar to the sediments in Ozark springs and streams, and to isolate the bacteria from additional inputs and losses after initial inoculation. Figure 2 shows a survival chamber constructed from a 9 cm length of 5 cm diameter polyvinylchloride (PVC) pipe. The ends of the pipe are sealed with 0.45 µm membranes held in place by 3.8 cm trap adapters. The volume of the chambers was measured by addition of water, with an average volume of approximately 170 ml. The filter membranes allowed exchange of nutrients and water, but kept most bacteria confined within the chambers following inoculation and placement in the spring and stream environments. McFeters and Stuart (1972) and Bissonnette *et al.* (1975) constructed survival chambers of Plexiglas sealed with 0.45 µm membranes with a total internal volume of 20 ml. They used a hypodermic needle to subsample throughout the course of their study. The survival chambers used in the present study were inexpensive enough to allow each chamber to be used one time. Triplicate samples were collected over 14 retrievals, including the inoculated chambers and triplicate noninoculated chambers. Thus, once placed in the stream and spring, the chambers were allowed to remain in place until retrieval and delivery to the laboratory.

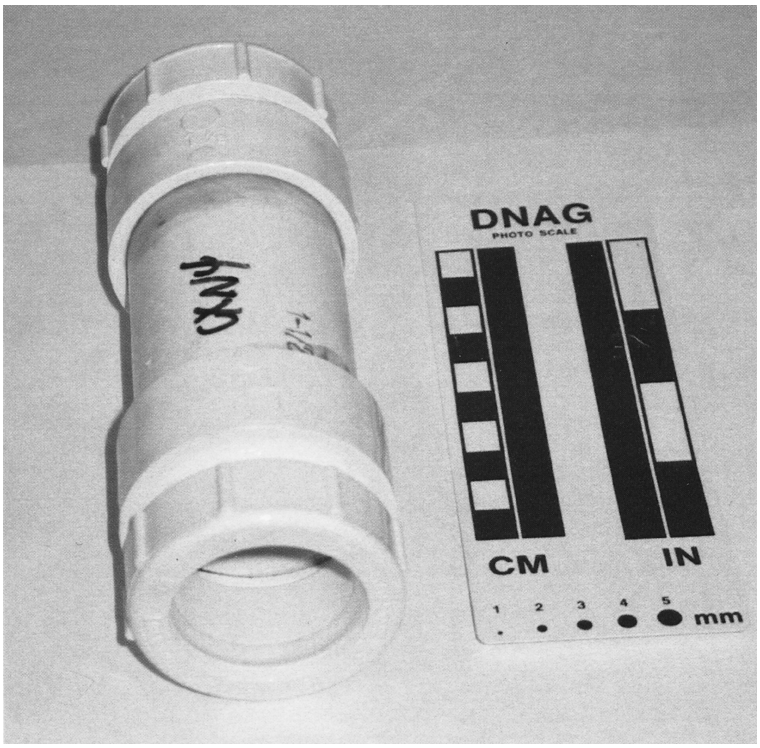

Figure 2. Survival Chambers.

Each chamber was loaded with 130 g of moist sediment collected from the floodplain deposits of the Illinois River. The average moisture content of the soil prior to placement in the chambers was 20 percent, based on moisture content determination of 10 subsamples. This provided about 100 g of soil on a dry weight basis in each chamber. The sediment used in the chambers was analyzed and found to contain less than 20 MPN *E. coli*/100 g. This was considered negligible in comparison to inoculated concentrations of 2.5 x 10^7 organisms/100 g sediment. The initial concentration in the chambers was determined by analyzing a time zero set of three inoculated chambers and three noninoculated chambers prior to transport to the field.

The sediment loaded chambers were either designated as control (no inoculation) or inoculated. The control chambers were topped off with sterile deionized water, sealed, and placed in coolers prior to transport to the field. The remainder of the chambers were inoculated with 2.5 x 10^7 organisms/100 g sediment of a nonpathogenic strain of *E. coli* (11775) obtained from a scientific supply company. The *E. coli* were grown in tryptic soy broth for 24 hours at 35˚C prior to inoculation of the chambers and transferred to the sediment filled chambers by pipette. The chambers were then topped off with sterile deionized water, sealed, and placed in coolers prior to transport to the field.

The prepared chambers were transported to the field and placed in the spring and stream environments. Chambers in the spring were anchored by strings to a cable suspended above the deepest pool within 2 m of the cave entrance. Random placement of the chambers in the pool helped eliminate potential bias that may have resulted if all replicates for each treatment had been placed side-by-side at the study site. The chambers were placed so they were fully submerged in water and rested on the sediments at the base of the pool. In the stream, the chambers were placed randomly in holes in concrete blocks that were wrapped with aluminum screen and placed in a pool in the flowing main channel of the river. The screen allowed water to flow freely through the concrete blocks but provided a barrier to transport of the chambers downstream. The blocks were cabled together and secured to two trees on the stream bank to minimize potential loss of the samples during high flow.

The chambers were retrieved initially at one-day intervals for the first four days, gradually expanding to a 12-day retrieval interval for the last chambers retrieved on the 75th day. Upon retrieval from the field, the chambers were placed in coolers and delivered to the laboratory for analysis. On sample collection days, water temperature was recorded and a water sample was collected from the spring and the stream for *E. coli* analysis. No other water quality parameters were analyzed during this study. Data for other water quality parameters were obtained from historic water samples collected at these sites and other similar settings throughout northwest Arkansas.

Once in the laboratory, the sediment in the chambers was carefully unloaded into sterile beakers. The chambers were rinsed with sufficient deionized water to bring the total volume of the sediment water slurry in the beaker to 200 ml. This mixture was stirred with a sterilized magnetic stir bar, making the slurry homogenous. Representative samples were obtained by subsampling this slurry with a pipette from about the midpoint of the beaker. These subsamples were then analyzed by the Most Probable Number (MPN) method to determine the concentration of total coliform and *E. coli* in the slurry (Elmund *et al.,* 1999). The collected water samples were also analyzed using the MPN technique. The MPN counts are statistical best estimates obtained by culturing a number of sample dilutions. These estimates are based on the principle of dilution to extinction. As an example, if a single, 1 ml aliquot from each of a series of 1:10 dilutions is examined and growth occurs at a dilution of 10^{-3} but not at 10^{-4}, the best estimate of the count is 10^3 bacteria per milliliter. By increasing the number of 1 ml aliquots examined at each dilution, a better estimate of the count can be obtained (WHO, 1996). A minimum of three dilutions per sample were made during this investigation. *E. coli* were enumerated in the samples using the QuantiTray-Colilert system (IDEXX Laboratories, Inc., Westbrook, Maine). This technique permits simultaneous measurement of total coliforms and *E. coli* using the MPN method. The test uses a “Defined Substrate Technology" (DST), using indicator nutrients specific for coliforms and *E.coli.* The reaction produces a yellow color in the case of total coliforms and a blue fluorescence in the case of *E.coli*. The indicator reagent ONPG (O-Nitrophenyl-b-d-galacotpyranoside) is metabolized by the enzyme b-galactosidase, which is present in all coliforms, to produce a yellow color. The reagent MUG (4-Methumbelliferyl-b-d-glucoronide) is metabolized by the enzyme b-glucuronidase, which is specific to *E.coli*, to produce a compound that fluoresces in UV light (IDEXX, 2005). After incubation at 35˚C for 24 hours, clear cells are counted as negative for *E.coli*. Yellow cells are positive for total coliform and cells that fluoresce under UV light are positive for *E.coli*. The number of positive cells is referenced to a table and corresponding MPN counts of total coliform and/or *E.coli* per 100 ml are determined (Elmund *et al.,* 1999).

RESULTS

Three inoculated chambers and three control chambers were analyzed for each designated sample collection day. All three replications for each environment per day were averaged and used to determined standard error (Figure 3). The bacterial concentrations of the original inoculated chambers were approximately 2.5 x 10^7 MPN/100g sediment. The study lasted 75 days, and during that time there was a 4-log die-off in the chambers in the Illinois River, resulting in final concentrations of 3.5 x 10^3 MPN/100g sediment. For chambers placed in Copperhead Spring, there was a 5-log die-off over the same period, with final concentrations of 1.36 x 10^2 MPN/100g sediment. Native *E. coli* were present in the sediment used to load the chambers (about 20 MPN/100g sediment) and were observed in the control chambers. Background concentrations in the sediment used to load the chambers were not significant in terms of the total bacterial load added to the inoculated chambers (20 MPN/100 g sediment compared to 2.5 x 10^7 MPN/100 g sediment, which is less than 10^{-5} percent). However, based on analysis of uninoculated chambers, the ratio of background *E. coli* to those added during inoculation could have been as high as 0.5 percent, still relatively insignificant. The *E. coli* in the uninoculated control chambers underwent an approximately 1-log die-off for the period of the study, with a final concentration of less than 1 MPN/100 g sediment. It was also observed that there were native organisms in the water column where observed concentrations did not exceed 1,000 MPN/100 ml during the study period. At other times, observed concentrations from Copperhead Spring and the Illinois River have exceeded 10,000 MPN/100 ml water (Marshall *et al.,* 1998; Al-Rashidy, 1999). *E. coli* concentrations in the water outside the survival chambers did increase prior to or coincident with storm pulses that occurred during the period of the study. However, the bacteria in the chambers show no effect from the storm runoff, indicating no significant exchange of bacteria between those contained in the survival chambers and bacteria external to the chambers, consistent with the chambers functioning as intended.

DISCUSSION AND CONCLUSION

Previous studies indicated that *E coli* can survive in various substrates for periods from three days to 268 days with temperatures from 4°C to 35°C (Flint, 1987; Van Donsel, 1967; Teague *et al.,* 1995;

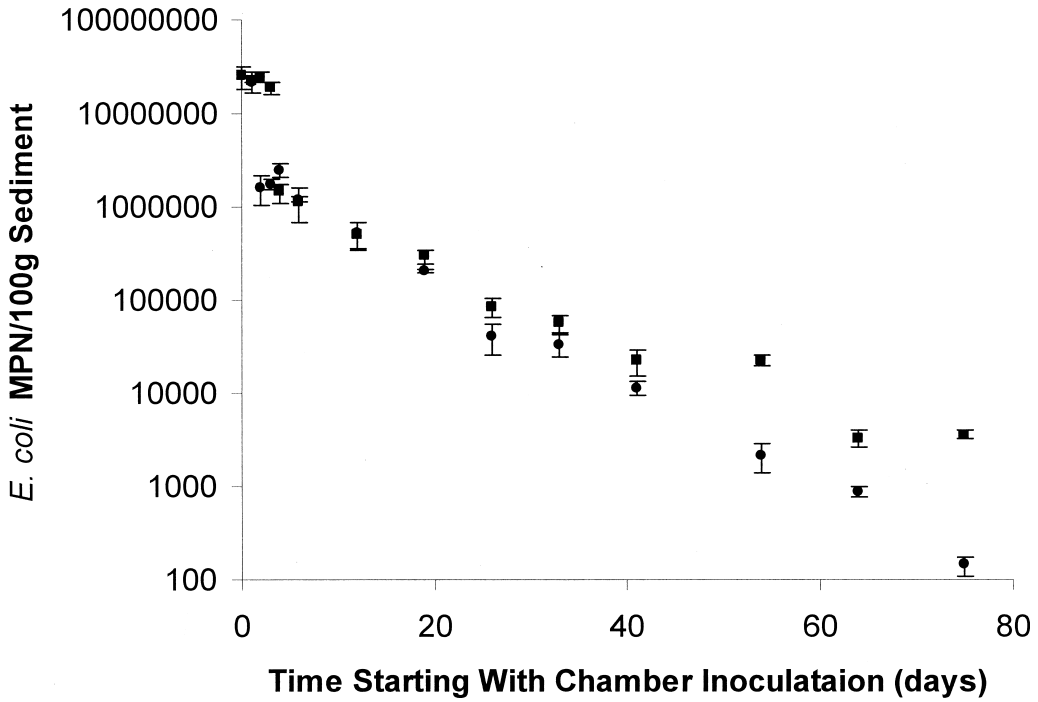

Figure 3. Bacterial Die-Off Curves for Illinois River and Copperhead Spring. Initial placement of survival chambers in the Illinois River occurred on November 10, 1999. Squares depict Illinois River. Circles depict Copperhead Spring. Standard error shown with error bars based on three replicates for each sample.

Schumaker, 2003). The results indicate *E. coli* can survive in the winter months for at least 75 days in sediment filled chambers placed in the water of Ozark stream and spring environments with no additional organisms from surface runoff or other sources. This is likely a conservative estimate of the survival time because the design of the chambers and placement at the study site provide an experiment that was an intermediate between a pure laboratory study and assessment of real field conditions. Placement at the site provided the opportunity for the exchange of fluids, and the fluctuation of temperature that occurred over the period of the study. Replicating this in the laboratory would have been very difficult. However, the design did not allow exchange of potential predators and may have limited availability of sediment bound nutrients. The exclusion of predators would tend to enhance the survival rate, while the limited availability of sediment bound nutrients would increase the die-off rate. Chapelle (2001) lists five separate functional components needed for culture media including a carbon source, a nitrogen source, inorganic ions – phosphate, potassium, sulfur, and trace metals, appropriate vitamins, and an appropriate electron acceptor. Two sources of carbon available in the sealed chambers include the total organic carbon (TOC) in the soil, and the TOC and dissolved organic carbon (DOC) in the water. Laincz *et al.* (2004) collected TOC and DOC from shallow lysimeters and from the springs at the site that show TOC and DOC in the soil water to be 24.6 and 22.9 mg/l, and TOC and DOC from a spring to be 2.5 and 2.1 mg/l, respectively. These data indicate that sufficient carbon was available in the sediment in the chambers, and in the water that moved through the chambers, to sustain the *E. coli*. During low flow, the average values for springs throughout northwest Arkansas range from 0.2 to 1.9 mg/l TOC. High flow values range from 1.8 to 6.9 mg/l TOC. Extremes during these periods include 0.09 mg/l TOC and 12.7 mg/l TOC (Davis *et al.,* 2000). Nitrate-nitrogen concentrations at the site range from about 2 mg/l to over 30 mg/l (Brahana *et al.,* 1999). Typical nitrate-nitrogen concentrations observed at other springs throughout northwest Arkansas range from about 2 to 7 mg/l (Davis *et al.,* 2000). Soluble reactive phosphorus (SRP) at the site ranges from 0.01 mg/l to over 1 mg/l, which is also very typical for springs throughout northwest Arkansas. Significant trace metals in the spring water include Fe, Zn, and Cu. The water chemistry provides an array of electron acceptors. The pH of the water generally varies between 6.5 and 7.5.

Trend analysis of the bacterial concentrations from the inoculated chambers from Copperhead Spring and the Illinois River resulted in the following exponential equations as a best fit (Figure 4). A power regression was also done but did not fit the data as well as the exponential equations. The power regression resulted in $R^2 = 0.56$ for both Copperhead Spring and

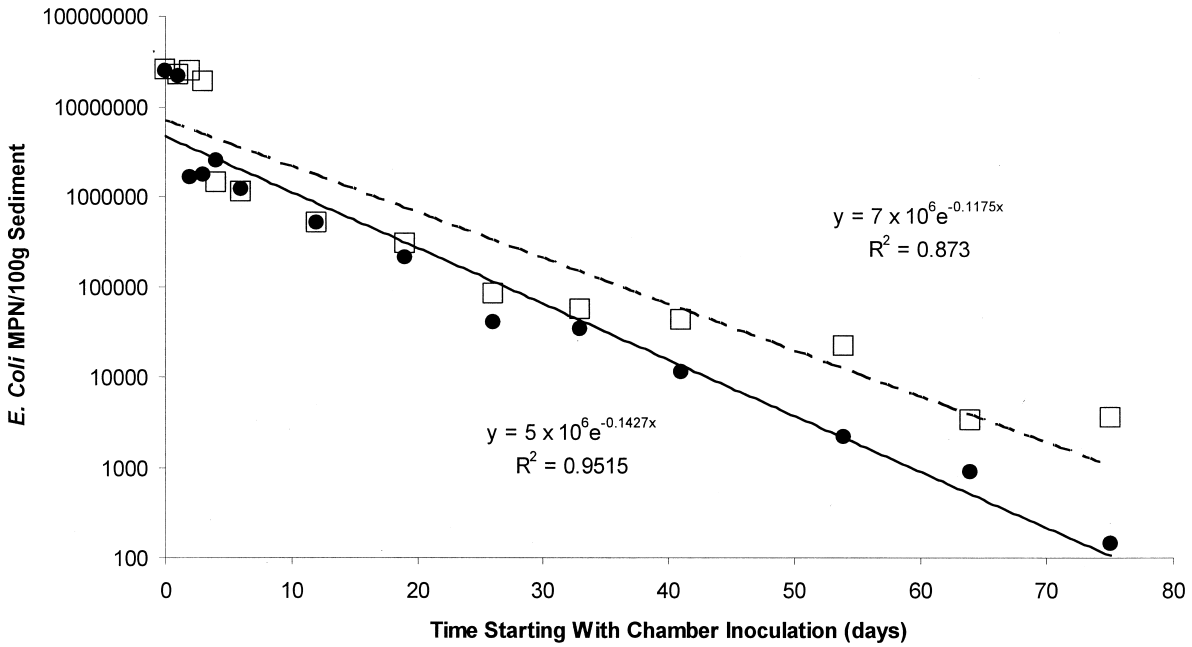

Figure 4. Trend Analysis of Bacterial Die-Off for Copperhead Spring (shown as solid circles) and Illinois River (shown as open squares) Sites. Data points represent average of three replicates.

the Illinois River data sets, and significantly overpredicted concentrations of *E. coli* below 10,000 MPN/100 g sediment.

Copperhead Spring

$$C_t = (5 \times 10^6)e^{-0.1427t}; \qquad R^2 = 0.96 \qquad (1)$$

Illinois River

$$C_t = (7 \times 10^6)e^{-0.1175t}; \qquad R^2 = 0.87 \qquad (2)$$

where C_t is the concentration (MPN *E. coli*/100 g sediment) at time t (days).

Extrapolation of the best-fit regression line for each environment indicates that the bacteria concentration would reach 1 MPN/100 g sediment at Copperhead Spring in about 105 days, and about 135 days at the Illinois River site. Based on this, it is concluded that bacteria can survive in these Ozark environments for at least four months with no fresh external inputs. Extended dry periods do occur in the area where no significant influx of bacterial from surface runoff or ground water recharge would be expected. However, these rarely last longer than a month or two at most. The longest period would be during the dry season, which generally begins in early July and extends through mid-October to late October. During this three to four month period, it is possible for surface runoff and ground water recharge to be very low, thus limiting input of bacteria from surface sources. It is noted however that in most years storm induced runoff and ground water recharge occur sporadically, except during times of extreme drought.

Survival of *E. coli* is dependent on environmental stresses including temperature, salinity, pH, nutrient concentrations, effects of solar radiation, and predation (Mitchell and Chamberlain, 1978; Tate, 1978; Burton *et al.*, 1987; Rozak and Colwell, 1987; Chapelle, 2001; Banning *et al.*, 2003). The pH in the Illinois River and Copperhead Spring generally ranges from about 6.5 to 7.5 and likely causes little stress to the *E. coli*. Whereas no direct measurement of salinity was made, the specific conductance of the water ranges from around 50 µS/cm to around 250 µS/cm, indicating very dilute conditions throughout the experiment. Copperhead Spring is isolated from direct solar radiation so this factor should not play a significant role in survival of the *E. coli* either, whereas the concrete blocks helped shade the chambers that were placed in the Illinois River. This leaves temperature, nutrient supply, and predation as the main factors influencing survival of *E. coli* in environments represented by this study. Mitchell and Chamberlin (1978) state two of the main factors influencing survival of bacterial organisms are predation and nutrients. Davies and Bavor (2000) indicate that predation is a major factor influencing bacterial survival, suggesting that the key to bacterial longevity at their study site was related to adsorption of bacteria to fine particles that protect them from predators. Sibille (1998) suggests that protozoan grazing may account for loss of *E. coli* in drinking water distribution systems. As indicated previously, carbon and nutrient concentrations from the Springfield Plateau aquifer in the vicinity of SEW provide evidence that there are sufficient nutrients (carbon and nitrate) in the environment to sustain the bacteria. This leaves predation and temperature as the main factors influencing *E. coli* survival in the chambers. No data are available for potential predators in the chambers, but external sources of predators were excluded by the 0.45 µm filter paper on the end of the chambers.

Of greater importance is the water temperature. *E. coli* need the warmth of animal intestines to sustain maximum reproduction. It is probable that the cooler temperatures slowed the metabolism of the organisms, thereby prolonging their existence (Chapelle, 2001; Cullimore, 1993). As noted previously, other investigators indicate longer survival times at cooler temperatures.

The temperatures in Copperhead Spring remained fairly stable (15°C to 9°C) with an average temperature of 12°C. The Illinois River fluctuated more during the study, with high values of 17°C and a low of 3°C. The average temperature for the river was 10°C. Interestingly, the die-off was greater in the Copperhead than in the Illinois. This could be due to the cooler seasonal temperatures in the river slowing the metabolism of the *E. coli* during the last 65 days of the study. If this study were done in the summer months, the results would likely be reversed. Copperhead would have a lower *E. coli* die-off rate than the Illinois River because the water temperatures in the river change significantly from season to season. Considering this, results from this study are primarily applicable to low temperature periods, and relatively constant temperature environments like the springs.

Because of the unique geology of the area, shallow aquifers have less restricted flow paths from the surface. This is environmentally undesirable owing to the high probability of contamination. The spreading of poultry litter on fields is a common practice in this area. If *E. coli* from animal manures infiltrates the aquifers via surface runoff, the water supply may become contaminated. Unfortunately, applied animal manures are not the only concern. Human waste from on-site disposal systems is also a major problem.

Most of northwest Arkansas is rural and many (over 90 percent) homes have on-site disposal systems. If these tanks rupture, leak, or otherwise fail to perform as designed, then the ground water may become contaminated. The survival of fecal coliform bacteria, including *E. coli*, in these environments over extended periods highlights a continuing human health hazard associated with consumption of nontreated water from wells, springs, and streams from the mantled karst of the Ozark region. It also suggests the potential for other surface derived pathogens to survive for similar periods in these environments.

ACKNOWLEDGMENTS

This project was supported in part by a grant from the U.S. Geological Survey through the Water Resources Research Institute Program 104 B Funds.

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